KETOGULONIGENIUM SHUTTLE VECTORS

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CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This non-provisional application is related to provisional application numbered 60/194,625, filed April 5, 2000, the content of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates, in general, to vectors comprising a *Ketogulonigenium* replicon. More specifically, the present invention relates to vectors comprising a *Ketogulonigenium* replicon found on the endogenous plasmid contained in Deposit No. NRRL B-30035.

Background Information

[0003] The exploitation of microorganisms to synthesize vitamin C or its chemical pathway intermediates has both economic and ecological advantages. One key intermediate in vitamin C synthesis is 2-keto-L-gulonic acid (2-KLG), which is easily converted chemically to L-ascorbic acid (vitamin C) by esterification followed by lactonization (Delic, V. *et al.*, "Microbial reactions for the synthesis of vitamin C (L-ascorbic acid," in *Biotechnology of Vitamins, Pigments and Growth Factors*, Vandamme, E.J., ed., Elsevier Applied Science (London & New York) pp. 299-336 (1989)). Members of a number of bacterial genera have been identified that produce 2-KLG from the oxidation of sorbitol. Such 2-KLG producing genera include the acidogenic, alpha-proteobacteria

Gluconobacter and *Acetobacter*, the gamma-proteobacteria *Pseudomonas*, *Escherichia*, *Klebsiella*, *Serratia* and *Xanthmonas*, the Gram positive *Bacillus*, *Micrococcus* and the unofficial genus *Pseudogluconobacter* (Imai, K. *et al.*, U.S. Patent No. 4,933,289 (1990), Sugisawa, H. *et al.*, "Microbial production of 2-keto-L-gulonic acid from L-sorbose and D-sorbitol by *Gluconobacter melanogenus*," *Agric. Biol. Chem.* *54*:1201-1209 (1990), Yin, G. *et al.*, U.S. Patent No. 4,935,359 (1990) and Nogami, I. *et al.*, U.S. Patent No. 5,474,924 (1995)).

[0004] To aid in increasing the yield of bacterial products, attempts have been made to exploit endogenous plasmids within microorganism strains. For example, shuttle vectors derived from endogenous plasmids have been utilized in an attempt increase production of bacterially generated compounds in *Gluconobacter* and *Acetobacter* (Beppu, T. *et al.*, U.S. Patent No. 5,580,782 (1996), Fujiwara, A. *et al.*, U.S. Patent No. 5,399,496 (1995), Tonouchi *et al*, U.S. Patent No. 6,127,174 (2000), Hoshino, T. *et al.*, U.S. Patent No. 6,127,156, (2000)).

SUMMARY OF THE INVENTION

[0005] The present invention provides an isolated or purified nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a nucleotide sequence of a *Ketogulonigenium* plasmid replicon found on the endogenous plasmid contained in Deposit No. NRRL B-30035 (Deposited July 21, 1998, Agriculture Research Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois 61604, U.S.A.). The invention further provides host cells transformed with the vector of the present invention. The invention also provides a method of producing polypeptides and/or antisense transcripts by culturing host cells transformed with the vector of the present invention.

[0006] Further advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] ***Figure 1*** shows the nucleotide (SEQ ID NO:1) sequence of a replicon of the endogenous plasmid contained in *Ketogulonigenium* strain NRRL Deposit No. B-30035. The nucleotide has a sequence of about 2112 nucleic acid residues.

[0008] ***Figure 2*** shows the nucleotide (SEQ ID NO:2) sequence of the endogenous plasmid determined by sequencing of the endogenous plasmid contained in NRRL Deposit No. B-30035. The nucleotide has a sequence of about 8509 nucleic acid residues.

[0009] ***Figure 3*** shows the nucleotide (SEQ ID NO:3) sequence of a shuttle vector plasmid contained in NRRL Deposit No. B-30434 (Deposited March 27, 2001, Agriculture Research Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois 61604, U.S.A.). The nucleotide has a sequence of about 5859 nucleic acid residues.

[0010] ***Figure 4*** shows the nucleotide (SEQ ID NO:4) sequence comprising a region that supports plasmid vector replication in *Ketogulonigenium* host species. The nucleotide has a sequence of about 2517 nucleic acid residues.

DETAILED DESCRIPTION OF THE INVENTION

[0011] Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the ABI Prism 3700). Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined

by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule.

[0012] Unless otherwise indicated, each "nucleotide sequence" set forth herein is presented as a sequence of deoxyribonucleotides (abbreviated A, G , C and T). However, by "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U) where each thymidine deoxynucleotide (T) in the specified deoxynucleotide sequence in is replaced by the ribonucleotide uridine (U). For instance, reference to an RNA molecule having the sequence of SEQ ID NO:1 set forth using deoxyribonucleotide abbreviations is intended to indicate an RNA molecule having a sequence in which each deoxynucleotide A, G or C of SEQ ID NO:1 has been replaced by the corresponding ribonucleotide A, G or C, and each deoxynucleotide T has been replaced by a ribonucleotide U.

[0013] As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

[0014] By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the DNA

molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

[0015] In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, in the endogenous plasmids contained in NRRL Deposit No. B-30035. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers.

[0016] Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (e.g., the deposited endogenous plasmid), for instance, a portion 50-750 nt in length, or even to the entire length of the reference polynucleotide, also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequence of the deposited DNA or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1). By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide, (e.g., the deposited DNA or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1)). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in Molecular Cloning, A Laboratory Manual, 2nd. edition, edited by Sambrook, J., Fritsch, E. F. and Maniatis, T., (1989), Cold

Spring Harbor Laboratory Press, the entire disclosure of which is hereby incorporated herein by reference.

[0017] By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the pyruvate carboxylase polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence.

[0018] As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1 or to the nucleotide sequence of the deposited endogenous plasmid can be determined conventionally using known computer programs such as the FastA program. FastA does a Pearson and Lipman search for similarity between a query sequence and a group of sequences of the same type nucleic acid. Professor William Pearson of the University of Virginia Department of Biochemistry wrote the FASTA program family (FastA, TFastA, FastX, TFastX and SSearch). In collaboration with Dr. Pearson, the programs were modified and documented for distribution with GCG Version 6.1 by Mary Schultz and Irv Edelman, and for Versions 8 through 10 by Sue Olson.

[0019] The present invention provides an isolated or purified vector comprising a nucleic acid molecule comprising a nucleotide sequence of a *Ketogulonigenium* replicon found on the endogenous plasmid contained in Deposit No. NRRL B-30035 (ADM 291-19), pADM291, and at least one exogenous nucleotide sequence. In an additional embodiment of the invention, the *Ketogulonigenium* replicon comprises the nucleotide sequence in SEQ ID NO:1.

[0020] As used herein, an exogenous nucleotide sequence is a nucleotide sequence which is not present in the native or wild type endogenous plasmid contained in Deposit No. NRRL B-30035. However, an exogenous nucleotide sequence also includes a sequence endogenous to the plasmid contained in Deposit No. NRRL B-30035, but mutated or non-natively regulated.

[0021] *Ketogulonigenium* (Ke.to.gu.lo.ni.gen'.i.um. M.L. n. *acidum ketogulonicum* ketogulonic acid; Gr. v. *gennaio* to produce; M.L. n. *ketogulonigenium* ketogulonic acid producing) is gram negative, facultatively anaerobic, motile or non-motile, has ovoid to rod-shaped cells, 0.8-1.3 μm long, 0.5-0.7 μm in diameter, with tapered ends, occurring as single cells, pairs and occasionally short chains. Some strains form elongated cells (up to 30 μm in length) on TSB. Flagella and fimbrae have been observed. Colonies are tan colored, smooth, circular, entire, raised to convex, 1-2 mm in diameter with a diffusable brown pigment after 48 hrs incubation. Oxidase and catalase reactions are positive. Optimum temperature range is 27 to 31 °C, optimum pH range is 7.2 to 8.5 and optimum Na^+ concentration is 117-459 mM. Chemoorganotrophic. Carbon sources utilized include arabinose, cellobiose, fructose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, rhamnose, sorbitol, sorbose, sucrose, trehalose, pyruvate and succinate. Favored carbon sources are inositol, mannitol, glycerol, sorbitol, lactose and arabinose. All strains examined produce 2-keto-L-gulonic acid from L-sorbose. Major cellular fatty acids are 16:0 and 18:1 ω7c/ω9t/ω12t and the mol% DNA G+C is 52.1 to 54.0 percent. Small subunit rDNA sequence analysis place this genus in the alpha subgroup of the Proteobacteria. All strains isolated in the present study group originated in soil. DNA reassociation studies divide the genus into two species. *K. vulgarae* and *K. robustum*, of which *K. vulgarae* is the designated type species. A group of bacteria having the above-mentioned properties does not belong to any known genera as described in Bergey's Manual of Systematic Bacteriology, and therefore belongs to a new genus.

[0022] The present invention further provides vectors which have a replicon functional in *Escherichia coli* (*E. coli*) and in *Ketogulonigenium*. *E. coli* is known to be an efficient host for amplification of a vector DNA and manipulation of recombinant DNA by simple and rapid methods. On the other hand, *Ketogulonigenium* can be used as a host for expression of *Ketogulonigenium* genes. Since the vectors of the present invention are such functional constructs, they enable cloning of certain genes of *Ketogulonigenium* in *E. coli* and thereafter the effective expression of the genes in *Ketogulonigenium*. Furthermore, it is favorable that such functional constructs also contain a DNA region necessary for conjugal transfer (mob site). Hence the vectors of the present invention can first be assembled in *E. coli* and then directly introduced into *Ketogulonigenium* by conjugal mating without isolation of plasmid DNA from *E. coli*.

[0023] Polynucleotides of interest may be joined to a vector containing a selectable marker for propagation in the host. A plasmid vector can be introduced by transformation mediated by calcium chloride or other cation salts, electroporation and other transformation methods, viral or phage transduction or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, "Basic Methods in Molecular Biology," (1986), and, J. Sambrook, E.F. Fritsch and T. Maniatis (1989) "Molecular Cloning: A Laboratory Manual", 2nd Ed..

[0024] Preferred are vectors comprising cis-acting control regions to a polynucleotide of interest. Appropriate *trans* acting factors may be supplied by the host, supplied by a complementing vector, or supplied by the vector itself upon introduction into the host.

[0025] In certain preferred embodiments in this regard, the vectors provide for specific expression, which may be inducible, mutant-specific and/or condition-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature, nutrient additives or chemical additives. Other suitable environmental factors will be readily apparent to the skilled artisan.

[0026] Expression vectors useful in the present invention include chromosomal-, episomal-vectors e.g., vectors derived from plasmids, bacteriophage, and vectors derived from combinations thereof, such as cosmids and phagemids.

[0027] A DNA insert of interest should be operatively linked to an appropriate promoter which is preferably a host-derived promoter. Preferably, the host-derived promoter is positioned in front of a polylinker insert such that transcripts initiated at the promoter cause expression of genes cloned into the polylinker. The expression constructs can further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs can include a translation initiating codon appropriate for the host at the beginning and a termination codon appropriately positioned at the end of the coding sequence to be translated.

[0028] The invention further provides the vectors of the present invention comprising a peptide fusion cloned gene expression system whereby a fused peptide serves as a tag for detecting and/or isolating the cloned gene product. The invention also provides a fused short peptide containing a biocytin residue (lysine covalently modified with a biotin), so that avidin-sepharose columns can be used to isolate the protein, and avidin-conjugated alkaline phosphatase can be used to detect the protein fusion on Western Blots.

[0029] In an additional embodiment, the DNA insert of interest additionally comprises a nucleotide sequence encoding a His-Tag sequence downstream of the promoter and polylinker, such that genes cloned into the polylinker can form translational fusions with the His-Tag. In this way, isolation of cloned proteins is facilitated because the fused His-Tag sequence allows rapid purification. A His-Tag is a polyhistidine sequence that binds to Nickel-Agarose columns, thus allowing rapid purification of the fusion protein from crude cell extracts. (Chen, B.P and Hai, T., *Gene 139*:73-79 (1994)). Removal of the His-Tag from the cloned polypeptide is achieved by treating the purified protein preparation with factor Xa or other suitable proteases.

[0030] In an additional embodiment, the DNA insert of interest additionally comprises nucleotides encoding a polypeptide. The polypeptide can be a small, immunogenic peptide sequence of about 5 to 30 amino acids, and preferably from about 10 to 20 amino acids. Ideally, this peptide sequence is not expressed natively in *Ketogulonigenium*. The DNA insert is adjacent to and translationally fused to the polylinker on either side, such that genes cloned into the polylinker are expressed as protein fusions with the immunogenic peptide. The fused peptide-antibody interaction can be used in protein isolation schemes and for detecting expression of the cloned gene fusion by immunoblotting of the expressed protein. (Enomoto, S. *et al.*, *Biotechniques 24*:782-788 (1998)).

[0031] The invention further provides a functional gene expression vector for a periplasmic, outer membrane, or exported protein. (Payne, M.S. and Jackson, E.N., *J. Bacteriol. 173*:2278-2282 (1991)). Specifically, the invention provides a DNA insert in the vector of the insert further comprising a suitable N-terminal signal sequence from a periplasmic or exported protein gene (derived from *Ketogulonigenium* or other bacterial genera) inserted downstream of the promoter and upstream of the polylinker. In this way a periplasmic expression system is combined with easy recovery and purification of the cloned protein fusion.

[0032] The invention further provides a vector comprising a nucleotide molecule comprising a nucleotide sequence of a *Ketogulonigenium* replicon found on the endogenous plasmid of NRRL B-30035 (ADM 291-19) additionally comprising a sequence encoding a periplasmic thioredoxin-like function similar to that of the DsbA or *Pseudomonas* or *Bradyrhizobium* TlpA. The *dsb*A gene in *E. coli* encodes a protein that is able to catalyze formation and isomerization of disulfide bonds (Wunderlich, M. and Glockshuber, R, *J. Biol. Chem. 268*:24547-24550 (1993). Co-expression of *dsb*A, *tlp*A or the genes of functionally analagous proteins is sometimes necessary for functional expression of cloned periplasmic enzymes.

[0033] The invention further provides a vector comprising a nucleotide molecule comprising a nucleotide sequence of a *Ketogulonigenium* replicon found on the

endogenous plasmid of NRRL B-30035 (ADM 291-19) additionally comprising a DNA sequence that encodes an easily isolatable protein "handle" inserted adjacent to, and in translational fusion with either side of the polylinker, such that genes cloned into the polylinker are expressed as protein fusions between the cloned protein and the vector protein handle. In this way, purification of the protein products of the cloned gene are facilitated by applying well tested purification procedures for the "protein handle." More specifically, the cloned protein follows the handle protein during the purification process. After purification, the fusion protein is then separated into its two native protein products through the application of factor Xa or other suitable protease.

[0034] In one embodiment, the vectors of the present invention comprise a cosmid site which allows for making a DNA library with about 30 kilobase inserts.

[0035] In an additional embodiment, the vectors of the present invention further comprises temperature-sensitive plasmid replication functions and regions of DNA homology to the host chromosome. The temperature sensitive replication functions allow control of integration into the homologous regions of the host chromosome through manipulation of temperature.

[0036] In one embodiment, the vectors of the present invention include two different antibiotic resistance genes inserted into two different restriction sites in the vector. One or both of the antibiotic resistance genes can carry a restriction site for gene cloning. In this way, one resistance marker is used to select transformants, and the other is used to screen for cloned DNA insertions by insertional inactivation. Suitable marker genes for the vectors of the present invention are all antibiotic resistance genes which are expressed in *E. coli* or *Ketogulonigenium*. Preferred antibiotic resistance genes include amikacin, ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin, spectinomycin, streptomycin or tetracycline resistance genes. Particularly preferred markers include ampicillin, chloramphenicol, erythromycin, kanamycin,

penicillin, spectinomycin, streptomycin, and/or tetracycline. Other suitable markers will be readily apparent to the skilled artisan.

[0037] Suitable replicons functional in *E. coli* for the vectors of the present invention can be selected from the group comprising DNA fragments containing a replicon of *E. coli*, or of any plasmid or phage which can autonomously replicate in *E. coli*. Such replicons may be isolated from the group comprising plasmids RP4, RSF1010, and pSUP301 (U.S. Patent No. 5,399,496) lambda phages, such as phage lambda ATCC 10798, P1 phages such as P1 ATCC 25404-B1 or T-coliphages, such as coliphage T4 ATCC 11303-B4. Other suitable plasmids and replication origins will be readily apparent to the skilled artisan.

[0038] A suitable replicon functional in *Ketogulonigenium* for the vectors of the present invention is any DNA fragment which can support autonomous replication in *Ketogulonigenium.* Preferred are DNA fragments selected from the group comprising a replicon of *Ketogulonigenium* or of any plasmid, endogenous or otherwise, or phage which can autonomously replicate in *Ketogulonigenium.*

[0039] The mob site includes the origin of transfer (*ori*T) and acts as a recognition site for certain *trans* active plasmid transfer functions (R. Simon *et al.*, *Bio/Technology 1*:784-791 (1983). A mob region is usually a *mob* gene and an *ori*T from any conjugation plasmid. The mob site can be obtained from a conjugative plasmid, e.g., plasmids RK2, RP4, RSF1010, or plasmids belonging to incompatibility groups IncP, IncQ, IncC, IncB, IncF, IncG, IncI, IncK, IncM, IncN, IncPa, IncPb, IncW, IncX, and IncZ or their derivatives. Other suitable conjugative plasmids will be readily apparent to the skilled artisan. The mob site containing plasmid can be transferred from its original host to another host with the help of *tra* genes by using bi-parental conjugal or tri-parental conjugal mating. The *tra* genes are well known as transfer genes of the broad-host-range conjugative plasmids. In the tri-parental conjugal mating, a donor strain harboring mob site-containing plasmids is mixed with a strain harboring plasmids containing *tra* genes, such as RP4 and RK2, and with a recipient strain.

Conjugation provides an alternative method, for example, compared to transformation, of placing vectors and other genetic material into the cell. Other suitable methods will be readily apparent to the skilled artisan.

[0040] The vectors of the present invention may also comprise one or more further nucleotide molecules (also called "inserts"), for example DNA sequences having multicloning sites (also called "polylinker inserts"), expression control sequences, cos sites, terminator sequences, ribosome binding sites, DNA sequences encoding signal peptides and/or proteins, to add further desirable functions to the shuttle vector. Other suitable inserts will be readily apparent to the skilled artisan.

[0041] In more detail, the vectors of the present invention may comprise DNA sequences including one or more multicloning sites (Messing *et al.*, *Methods in Enzymology 101*:20 (1983) derived from a variety of plasmids and phages, or from synthetic DNA sequences for convenient cloning. Suitable sources include pUC18 (Boehringer Mannheim), pUC19 (Boehringer Mannheim), M13mp8 (Boehringer Mannheim), and pBluescript. Other suitable multicloning sites will be readily apparent to the skilled artisan.

[0042] Furthermore, the vectors of the present invention may contain a wide variety of expression control sequences, such as the *E. coli lac*, *trp*, *tac* or beta-lactamase expression control system, control sequences of phage origin, such as the phage Lambda P_L promoter, or expression control sequences derived from *Ketogulonigenium* strains, to name a few. Other expression control systems will be known to the skilled artisan. In addition, the shuttle vectors may contain cos sites for *in vitro* packaging. Furthermore, the expression constructs will further optionally contain terminator sequences for transcription, initiation, termination, natural or synthetic ribosome binding sites for effective translation, DNA sequences encoding signal peptides for efficient localization of the cloned protein(s) and structural genes of marker proteins all of which are used to construct the efficient *Ketogulonigenium* host-vector systems of the present invention.

[0043] In another embodiment, the vector of the present invention may contain an *E. coli*, *Ketogulonigenium*, or other promoter from a suitable gene, in front of a promoterless reporter gene, such as β-galactosidase, β-glucuronidase, green fluorescent protein or α-amylase that has a polylinker inside the reporter gene. In this way, cloned nucleic acid inserts may be screened for by inactivation of the reporter function. Other suitable reporter genes will be readily apparent to the skilled artisan.

[0044] In another embodiment, pADM291 can contain a promoterless reporter gene such as β-galactosidase, β-glucuronidase, luciferase, green fluorescent protein, α-amylase, uroporphyrinogen III methyltransferase (cobA) from *Propionibacterium freudenreichii* inserted just adjacent to an inserted polylinker, such that DNA fragments cloned into the polylinker and comprising a transcriptional promoter can cause transcription and expression of the vector reporter function in the host. In this way, cloned inserts can be screened for those containing at least a transcriptional promoter that is active in the host organism.

[0045] In another embodiment, pADM291 can contain a reporter gene, such as β-galactosidase, β-glucuronidase, green fluorescent protein or α-amylase that lacks a promoter and a translation initiation site, inserted adjacent to a polylinker such that, nucleotide fragments cloned into the polylinker and comprising a promoter and translation initiation site can form a translational fusion with the reporter function and cause expression of the reporter function in the host. In this way, cloned inserts can be screened for those containing a transcriptional promoter and translation initiation site which are active in the host organism.

[0046] The vectors of the present invention can be obtained by the following steps using the materials as described in the present description and by using recombinant DNA techniques as described in the art and known to one of ordinary skill:

(a) Preparing a nucleotide sequence containing a marker gene;

(b) Preparing a nucleotide sequence containing a replicon functional in *E. coli*;

(c) Preparing a nucleotide sequence containing a mob site;

(d) Combining the nucleotides described in (a) through (d) along with the nucleotide of the present invention by digesting the said nucleotide sequences with an appropriate restriction enzyme and ligating them to obtain the recombinant shuttle vectors of the present invention.

[0047] By these steps, the vectors containing marker genes, a replicon functional in *E. coli*, a replicon functional in *Ketogulonigenium* and a functional mob site can be constructed.

[0048] In another embodiment the pADM291 nucleotide may be fused with another vector which replicates in *E. coli*, *Acetobacter*, *Corynebacterium*, *Bacillus*, *Rhodobacter*, *Paracoccus*, *Pseudomonas*, *Roseobacter*, *Pseudogluconobacter*, *Gluconobacter*, *Serriatia*, *Mycobacterium*, or *Streptomyces*. Preferred is fusion with a vector which replicates in *E. coli*, such as the pET vectors, pUC18, or pUC19. Other suitable vectors will be readily apparent to the skilled artisan.

[0049] The vectors of the present invention can be transferred from *E. coli* to *Ketogulonigenium* with a very high frequency by a conjugal mating without the isolation and purification of the vector DNA. With these vectors, a genomic library constructed in *E. coli* can be transferred into *Ketogulonigenium* in one experiment. Thus, the vectors of the present invention are highly efficient in view of their simplicity in cloning experiments.

[0050] Among vectors with which the the nucleotide sequence of a *Ketogulonigenium* replicon found on the endogenous plasmid contained in Deposit No. NRRL B-30035 may be combined include pA2, pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pUC18, available from Takara Shuzo Co., Ltd.; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

[0051] The vectors of the present invention incorporate a DNA sequence which confers upon the vector the capacity for autonomous replication in host *E. coli* cells.

[0052] The vector of the present invention may be combined with an *E. coli*-derived plasmid to create a vector which is able to replicate in both *Ketogulonigenium* and *E. coli*.

[0053] The present invention also provides a vector comprising a replicon functional in an organism selected from the genera comprising *Acetobacter*, *Corynebacterium*, *Bacillus*, *Rhodobacter*, *Paracoccus*, *Pseudomonas*, *Roseobacter*, *Pseudogluconobacter*, *Gluconobacter*, *Serriatia*, *Mycobacterium*, and *Streptomyces*. Other suitable genera will be readily apparent to the skilled artisan.

[0054] The plasmid may optionally contain its native expression vector and/or expression vectors which include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial endogenous plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

[0055] Among known bacterial promoters suitable for use in the present invention include the *E. coli lac*I and *lac*Z promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda P_R and P_L promoters and the *trp* promoter. Other suitable promoters will be readily apparent to the skilled artisan.

[0056] The invention also provides a shuttle vector comprising a *Ketogulonigenium* replicon found on the endogenous plasmid contained in Deposit No. B-30035 and at least one exogenous nucleotide sequence, wherein said shuttle vector autonomously replicates in *Ketogulonigenium* and at least one organism selected from the genera comprising *Acetobacter*, *Corynebacterium*, *Bacillus*, *Rhodobacter*, *Paracoccus*, *Roseobacter*, *Pseudomonas*,

Pseudogluconobacter, *Gluconobacter*, *Serratia*, *Mycobacterium*, and *Streptomyces*.

[0057] The invention also provides a method of transforming a host cell with the vectors of the present invention to obtain a stably transformed host cell. Such transformation can be effected by conjugation, including both biparental and triparental mating, transformation mediated by calcium chloride or other cation salts, electroporation and other transformation methods, transduction, or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, "Basic Methods in Molecular Biology," (1986) and J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). Preferred methods are conjugation and electroporation. Conjugation is a a process by which bacterial unilaterally transfer DNA from a donor to a recipient cell through cell to cell contact.

[0058] Suitable host cells comprise organisms selected from the genera comprising *E. coli, Ketogulonigenium*, *Acetobacter*, *Corynebacterium*, *Bacillus*, *Rhodobacter*, *Paracoccus*, *Roseobacter*, *Pseudomonas*, *Pseudogluconobacter*, *Gluconobacter*, *Serriatia*, *Mycobacterium*, and *Streptomyces*.

[0059] A stably transformed cell is a cell wherein a transgene (recombinant DNA) is transmitted to every successive generation. The vector transformed into the above-mentioned cells can optionally comprise a transgene. As used herein, a transgene is defined as a transplanted nucleotide sequence which is exogenous, or non-native, to the host. An exogenous nucleotide sequence, as used in the current context, is a nucleotide sequence which is not found in Deposit No. NRRL B-30035. Thus, the term exogenous nucleotide sequence is meant to encompass a nucleotide sequence that is foreign to Deposit No. NRRL B-30035, as well as a nucleotide sequence endogenous, or native, to Deposit No. NRRL B-30035 that has been modified. Modification of the endogenous nucleotide sequence may include, for instance, mutation of the native nucleotide sequence or any of its regulatory elements. As used herein, mutation is defined as any

change in the wild-type sequence of genomic or plasmid DNA. An additional form of modification may also include fusion of the endogenous nucleotide sequence to a nucleotide sequence that is normally not present, in relation to the endogenous nucleotide sequence. The transgene may be regulated by its normal promoter, or more commonly, by a promoter that normally regulates a different gene. The invention also provides a method for producing transformed *Ketogulonigenium,* comprising transforming *Ketogulonigenium* with a transgene, comprising, an endogenous *Ketogulonigenium* replicon. Preferably, the endogenous *Ketogulonigenium* replicon is contained in Deposit No. NRRL B-30035. The term replicon as used herein is meant to encompass a DNA sequence comprising those genes and gene expression control elements such as promoters and terminators, other DNA sequence features such as short sequence repeats (iterons), origins of plasmid replication (*ori* or *ori*V sites), or other DNA sequence features that are required to support the autonomous replication of a circular DNA molecule in a bacterial host (Chapter 122, pp. 2295-2324, in *Escherichia coli and Salmonella: Cellular and Molecular Biology,* 2nd Edition, Frederick C. Neidhardt, Ed., ASM Press (1996)). The requirements of a replicon can vary from as little as a short *ori* sequence in the case of plasmids that do not require their own replication proteins, to larger sequences comprising one or more plasmid-borne replication genes. The definition of a transformed cell, as used herein, is a cell where DNA has been inserted into a bacterial cell. The transformation of *Ketogulonigenium* may be transient or stable.

[0060] In an additional embodiment, the invention provides a method for producing a polypeptide comprising culturing a host cell comprising vectors of the present invention under conditions such that the polypeptide is expressed, and recovering the polypeptide. The translated polypeptide encoded by the DNA in the plasmid may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the

polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification.

[0061] The translated protein encoded by the DNA contained in the plasmid can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

[0062] Methods used and described herein are well known in the art and are more particularly described, for example, in R.F. Schleif and P.C. Wensink, *Practical Methods in Molecular Biology,* Springer-Verlag (1981); J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); P.B. Kaufman *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Boca Raton, Florida (1995); *Methods in Plant Molecular Biology and Biotechnology*, B.R. Glick and J.E. Thompson, eds., CRC Press, Boca Raton, Florida (1993); P.F. Smith-Keary, *Molecular Genetics of Escherichia coli*, The Guilford Press, New York, NY (1989); *Plasmids: A Practical Approach*, 2nd Edition, Hardy, K.D., ed., Oxford University Press, New York, NY (1993); *Vectors: Essential Data*, Gacesa, P., and Ramji, D.P., eds., John Wiley & Sons Pub., New York, NY (1994); *Guide to Electroporation and electrofusions*, Chang, D., *et al.*, eds., Academic Press, San Diego, CA (1992); *Promiscuous Plasmids of Gram-Negative Bacteria*, Thomas, C.M., ed., Academic Press, London (1989); *The Biology of Plasmids*, Summers,

D.K., Blackwell Science, Cambridge, MA (1996); *Understanding DNA and Gene Cloning: A Guide for the Curious*, Drlica, K., ed., John Wiley and Sons Pub., New York, NY (1997); *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Rodriguez, R.L., *et al.*, eds., Butterworth, Boston, MA (1988); *Bacterial Conjugation*, Clewell, D.B., ed., Plenum Press, New York, NY (1993); Del Solar, G., *et al.*, "Replication and control of circular bacterial plasmids," *Microbiol. Mol. Biol. Rev. 62*:434-464 (1998); Meijer, W.J., *et al.*, "Rolling-circle plasmids from Bacillus subtilis: complete nucleotide sequences and analyses of genes of pTA1015, pTA1040, pTA1050 and pTA1060, and comparisons with related plasmids from gram-positive bacteria," *FEMS Microbiol. Rev. 21*:337-368 (1998); Khan, S.A., "Rolling-circle replication of bacterial plasmids," *Microbiol. Mol. Biol. Rev. 61*:442-455 (1997); Baker, R.L., "Protein expression using ubiquitin fusion and cleavage," *Curr. Opin. Biotechnol._7*:541-546 (1996); Makrides, S.C., "Strategies for achieving high-level expression of genes in *Escherichia coli*," *Microbiol. Rev. 60*:512-538 (1996); Alonso, J.C., *et al.*, "Site-specific recombination in gram-positive theta-replicating plasmids," *FEMS Microbiol. Lett. 142*:1-10 (1996); Miroux, B., *et al.*, "Over-production of protein in *Escherichia coli*: mutant hosts that allow synthesis of some membrane protein and globular protein at high levels," *J. Mol. Biol. 260*:289-298 (1996); Kurland, C.G., and Dong, H., "Bacterial growth inhibited by overproduction of protein," *Mol. Microbiol. 21*:1-4 (1996); Saki, H., and Komano, T., "DNA replication of IncQ broad-host-range plasmids in gram-negative bacteria," *Biosci. Biotechnol. Biochem. 60*:377-382 (1996); Deb, J.K., and Nath, N., "Plasmids of corynebacteria," *FEMS Microbiol. Lett. 175*:11-20 (1999); Smith, G.P., "Filamentous phages as cloning vectors," *Biotechnol. 10*:61-83 (1988); Espinosa, M., *et al.*, "Plasmid rolling circle replication and its control," *FEMS Microbiol. Lett. 130*:111-120 (1995); Lanka, E., and Wilkins, B.M., "DNA processing reaction in bacterial conjugation," *Ann. Rev. Biochem. 64*:141-169 (!995); Dreiseikelmann, B., "Translocation of DNA across bacterial membranes," *Microbiol. Rev. 58*:293-316 (1994); Nordstrom, K., and Wagner, E.G., "Kinetic

aspects of control of plasmid replication by antisense RNA," *Trends Biochem. Sci.* *19*:294-300 (1994); Frost, L.S., *et al.*, "Analysis of the sequence gene products of the transfer region of the F sex factor," *Microbiol. Rev.* *58*:162-210 (1994); Drury, L., "Transformation of bacteria by electroporation," *Methods Mol. Biol.* *58*:249-256 (1996); Dower, W.J., "Electroporation of bacteria: a general approach to genetic transformation," *Genet. Eng.* *12*:275-295 (1990); Na, S., *et al.*, "The factors affecting transformation efficiency of coryneform bacteria by electroporation," *Chin. J. Biotechnol.* *11*:193-198 (1995); Pansegrau, W., "Covalent association of the traI gene product of plasmid RP4 with the 5'-terminal nucleotide at the relaxation nick site," *J. Biol. Chem.* *265*:10637-10644 (1990); and Bailey, J.E., "Host-vector interactions in *Escherichia coli*," *Adv. Biochem. Eng. Biotechnol.* *48*:29-52 (1993).

[0063] The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

[0064] All patents and publications referred to herein are expressly incorporated by reference.

EXAMPLES

EXAMPLE 1

Isolation of plasmid DNA from *Ketogulonigenium* and *E. coli* strains.

[0065] A fresh culture of *Ketogulonigenium* strain ADM291-19 (NRRL B-30035) was grown in 10 ml X6L medium (2% Mannitol, 1%Soytone, 1% Yeast Extract, 0.5% Malt Extract, 0.5% NaCl, 0.25% K_2HPO_4, pH 7.8). A fresh

culture of *E.coli* strain DH5αMCR harboring plasmid pUC19 DNA was grown in 10 ml of Luria Broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) with 100 μg/ml ampicillin. Both cultures were incubated overnight with orbital shaking at 250 rpm at 30 degrees C for *Ketogulonigenium* and 37 degrees C for *E. coli*. DNA was isolated using the Promega Wizard *Plus* Midipreps DNA Purification System (Madison, WI). The culture was centrifuged at 10,000 x g for 10 minutes at 4°C in a Sorval RC-5B centrifuge using the SS-34 rotor. The pellet was suspend in 3 ml of 50mM Tris-HCl, pH 7.5, 10mM EDTA, 100(g/ml RnaseA. Three ml of cell lysis solution (0.2M NaOH, 1%SDS) was added and mixed by inverting the tube, then three ml of neutralization solution (1.32M potassium acetate, pH4.8) was added and mixed by inverting the tube. The lysate was centrifuged at 14,000 x g for 15 minutes at 4(C in a SS-34 rotor, then the supernatant was carefully decanted to a new centrifuge tube. Ten ml of resuspension resin (40% isopropanol, 4.2M guanidine hydrochloride) was added to the supernatant fluid and mixed by swirling, then the mixture was into the Promega Wizard Midicolumn, which was connected a vacuum manifold. Vacuum was applied to pull the resin/DNA mixture completely into the midicolumn. The column was washed twice with 15 ml of column wash solution (95% ethanol, 80mM potassium acetate, 8.3mM Tris-HCl, pH 7.5, 40(M EDTA. The reservoir was removed from the midicolumn with a scissors, then the column was placed in a microcentrifuge tube and centrifuged at 10,000 x g for 2 minutes to remove any residual solution. The midicolumn was transferred to a new microcentrifuge tube and 300 (l of sterile dH_2O was applied. The tube was microcentrifuged at 10,000 x g for 20 seconds to elute to the DNA into solution. About 2.5 ug of pADM291 plasmid DNA was recovered. A similar procedure would be employed for other plasmids from *Ketogulonigenium* or *E. coli*, except that choice and concentration of selective antibiotics would be altered as suitable for the plasmid being isolated.

EXAMPLE 2

Isolation of strains ADM29101 and ADMX6L01, Nalidixic acid resistant mutants of *Ketogulonigenium* strains ADM291-19 (NRRL B-30035) and ADMX6L(NRRL B-21627), respectively.

[0066] Three ml of X6L medium (2% Mannitol, 1%Soytone, 1% Yeast Extract, 0.5% Malt Extract, 0.5% NaCl, 0.25% K_2HPO_4, pH 7.8) was inoculated with strain ADM291-19 (NRRL B-30035) and the culture was grown overnight in an orbital shaker at 30 degrees C, 250 rpm. 50 uL of the overnight culture was transferred to 3ml of X6L medium to which 1μl of a 1% aqueous solution of Ethidium bromide had been added. This culture was incubated overnight as before. 200μl of the second overnight culture was spread on a plate of X6L medium containing 1.3% agar plus 50μg/ml of nalidixic acid. The plate was incubated 2-3 days at 30°C until colonies appeared. Colonies growing on these plates were picked and naladixic acid resistant strains were purified by additional cycles of streak purification on X6L medium plates containing 50 μg/ml of nalidixic acid. A naladixic acid resistant strain was preserved as strain ADM 29101. The same procedure was used to create ADMX6L01 from ADMX6L.

EXAMPLE 3

Construction of *Ketoguonigenium* suicide plasmid vector pJND1000.

[0067] To invent an *E. coli/Ketogulonigenium* shuttle vector it was desireable that we first identify a subsequence from pADM291 that, when cloned into *E. coli* or other plasmid vectors, would support plasmid replication in *Ketogulonigenium* hosts. In order to do this, a plasmid vector that could replicate in *E. coli* and be transferred efficiently to *Ketogulonigenium*, but which cannot replicate in *Ketogulonigenium* was devised.

[0068] pJND1000 was constructed from segments of plasmids pUC19 (GenBank Accession No. M77789), pUC4K (GenBank Accession No. X06404), pFD288 (GenBank Accession No. U30830), and pFC5 (David M. Lonsdale et.al, 1995, "pFC1 to pFC7: A novel family of combinatorial cloning vectors.", Plant Molecular Biology Reporter 13[4]:343-345).

[0069] The ampicillin resistance gene (*amp*^{R}) was removed from pUC19 by digesting pUC19 DNA with restriction enzymes *Dra*I and *Ssp*I, separating the 1748 bp vector fragment from the smaller amp^R fragment and other fragments by gel electrophoresis, then recovering the 1748 bp fragment from a gel slice. A kanamycin resistance gene (*kan*^{R}) fragment from pUC4K was prepared by digesting pUC4K with restriction enzyme *Pst*I, treating the mixture with Klenow Fragment to produce blunt ends, separating the fragments by gel electrophoresis, then purifying the 1240 bp *kan*^{R} fragment from a gel slice. The isolated fragments from pUC19 and pUC4K were mixed and ligated with T4 ligase following the protocol of GibcoBRL technical bulletin 15244-2 (Rockville, MD) to produce "intermediate p1", an intermediate plasmid carrying pUC19 features and a *kan*^{R} gene. Strain *E. coli* DH5(MCR was transformed with the ligation mixture following an established protocol ("Fresh Competent *E. coli* prepared using $CaCl_2$", pp. 1.82-1.84, *in* J. Sambrook, E.F. Fritsch and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual,* 2nd Ed.). Transformants were selected on Luria Broth plates containing 50μg/ml of kanamycin.

[0070] An *ori*T site for conjugative transfer was obtained from plasmid pFD288 by restricting it with *Hae*II, converting the single stranded ends to blunt ends by treating the mixture with Klenow Fragment, separating the fragments by agarose gel electrophoresis, then purifying the 778 bp *ori*T fragment from a gel slice. The intermediate p1 plasmid was opened at a single site with restriction enzyme *Sap*I, then treated with Klenow Fragment to convert the single stranded ends to blunt ends. The *Sap*I digested, blunt ended p1 intermediate was mixed with the purified *ori*T fragment, then treated with T4 ligase as above to create "intermediate p2", a plasmid which carries *ori*T in addition to *kan*^{R} and other

pUC19-derived features. Strain *E. coli* DH5αMCR was transformed with the ligation mixture as above. Intermediate p2 was confirmed by restriction digestion analysis.

[0071] The polylinker in intermediate plasmid p2 was replaced with the polylinker from pFC5. To do this the two plasmids were separately restricted with *Pvu*II, the fragments from each digestion reaction were separated by gel electrophoresis, then the pFC5-derived polylinker fragment (531 bp), and the larger non-polylinker fragment from intermediate p2 were purified from gel slices. The recovered p2 fragment and the pFC5-derived polylinker fragment were mixed and joined using T4 ligase as above to make plasmid pJND1000. The structure of pJND1000 was confirmed by restriction digestion analysis. PJND1000 has a functioning kanamycin resistance gene, an RK2-derived *ori*T site to permit conjugative transfer, a polylinker for DNA cloning, forward and reverse M13 primers to facilitate DNA sequenceing reactions into the polylinker, replicates in *E. coli* strains but not in *Ketogulonigenium* strains, and permits screening for cloned inserts by inactivation of *lac*Zα using Xgal indicator plates.

EXAMPLE 4

Cloning of DNA sequences from plasmid pADM291 into suicide vector pJND1000 to make *E. coli/Ketogulonigenium* shuttle vector candidates.

[0072] To find the functional replicon of pADM291, the Polymerase Chain Reaction (PCR) was used to generate various plasmid pADM291 DNA fragments flanked by restriction endonuclease recognition sites. These were cloned into the *Ketogulonigenium* suicide vector pJND1000 to make *Ketogulonigenium* shuttle vector candidates, which were transferred to *E. coli* hosts by plasmid transformation.

[0073] Forward and reverse oligonucleotide primers were designed with which to initiate PCR reactions using purified pADM291 DNA as substrate. The

oligonucleotide primers were prepared by a commercial lab (Sigma/Genosys, The Woodlands, TX) using established methods known in the art. The primers had the following features ("F"=forward primer; "R"=reverse primer):

Table I

Primer Label	DNA Sequence
Primer 1F	CGGAATTCGATCATATCATTCCCCAAGCGGAC
Primer 1R	GCTCTAGACGATGTACTCCTTGGTGCTCTCGAT
Primer 2F	CGGAATTCTGCTTCTTTTCGTTCGTTTCCGCC
Primer 2R	GCTCTAGAACATCGCTCATCTGTAGTCGCC
Primer 3F	CGG AATTCACAGTCAGGTGGCACATGTTCC
Primer 3R	CGG GATCCTGTGAAAAAGTGAGGACAGGCGGG
Primer 4F	CGGAATTCGGCAATGGGTCGAAATTCATAG
Primer 4R	CGGGATCCACGTTCCCTATTTTCCTCAATC
Primer 5F	CGGAATTCACACCGAAACACCTAACACGCAAG
Primer 5R	CGGGATCCAGTGCGGTTCACGTCATCAATG
Primer 6F	CGGAATTCTGCACTGCCGCTCTCGAAATG
Primer 6R	CGGAATTCACAAGATTGACGCAGCTCTTCGC
Primer 7F	CGGAATTCGGCAATGGGTCGAAATTCATAG
Primer 7R	CGCGGATCCACTTGTGTTGTCTTTCCC
Primer 8F	GCGGAATTCGACGCTGCAAACATCGAAAAAC
Primer 8R	CGGGATCCACGTTCCCTATTTTCCTCAATC

[0074] The PCR reactions generated pADM291 DNA fragments having the following endpoints, using the numbering of the DNA sequence in Figure 2 (SEQ ID NO2:).

Table II

PCR Fragment Label	Length	Fragment Endpoints	End Restriction Sites
PCR#1	2210 bp	8060 through 1760	5' *Eco*RI – *Xba*I 3'
PCR#2	1523 bp	876 through 2398	5' *Eco*RI – *Xba*I 3'
PCR#3	1222 bp	2017 through 3238	5' *Eco*RI – *Bam*HI 3'
PCR#4	2517 bp	2955 through 5471	5' *Eco*RI – *Bam*HI 3'
PCR#5	1965 bp	4704 through 6668	5' *Eco*RI – *Bam*HI 3'
PCR#6	2516 bp	5908 through 8423	5' *Eco*RI – *Eco*RI 3'
PCR#7	520 bp	2955 through 3474	5' *Eco*RI – *Bam*HI 3'
PCR#8	1065 bp	4407 through 5471	5' *Eco*RI – *Bam*HI 3'

[0075] PCR products were generated in a GENEAMP PCR System 9700 thermal cycler from Applied Biosystems using Gibco BRL *Taq* Polymerase (Rockville, MD), using 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds. Each reaction contained 1μl of a 50μM solution of each primer, 10ng of pADM291 template, 1.5mM $MgCl_2$ and PCR Buffer supplied with the *Taq* Polymerase. *Taq* polymerase and buffer were removed from the PCR products using the Promega Wizard DNA Clean-up Kit (Madison, WI). The pADM291 DNA fragments from each PCR reaction were prepared for ligation into plasmid pJND1000 by reaction with the appropriate endonuclease enzymes from Gibco BRL (Rockville, MD) as given in Table II (for example the PCR#1 products were digested with *Eco*RI and *Xba*I), followed by removal of enzyme and reaction buffer using Promega Wizard DNA Clean-up Kits. Likewise, for each PCR fragment a sample of purified plasmid pJND1000 DNA vector was digested with the same restriction enzymes that had been used to digest the PCR fragment, followed by enzyme and buffer removal using Promega Wizard DNA Clean-up Kits. Each PCR fragment was ligated into the pJND1000 vector in reactions utilizing T4 DNA ligase as in Example 3. A plasmid transformation was performed with each ligation product using competent *E. coli* strain DH5αMCR

as the recipient following the transformation protocol of Example 3. Transformants were spread on Luria Broth agar plates containing 50 ug/ml of kanamycin and 40 ug/ml of Xgal and grown at 37 deg C. Colonies that were white, indicating successful insertion of a PCR fragment into the pJND1000 vector, were picked and preserved, and a sample was transferred to 1 ml of Luria Broth containing 50 ug/ml kanamycin and incubated in an orbital shaker overnight at 37 deg C, 250 rpm. Plasmid DNA was prepared from each of the grown transformant cultures by the alkaline method of H.C. Birnboim and J. Doly (Nucleic Acids Research, 7:1513-1523 (1979)). The plasmids from these cultures, each of which was a candidate for a *Ketogulonigenium* shuttle vector, were named "p291-1" for PCR product #1, "p291-2" for PCR product #2, etc. Each plasmid was confirmed to be carrying the expected PCR insert via restriction digestion and gel electrophoresis.

EXAMPLE 5

Discovery of *Ketogulonigenium* shuttle vector p291-4 by conjugative transfer from *E. coli* hosts into *Ketogulonigenium* hosts and plasmid maintainance in *Ketogulonigenium* and bi-parental mating.

[0076] *Ketogulonigenium* shuttle vector plasmid candidates from Example 4 were introduced into *E. coli* strain S17-1 (ATCC 47055) by transformation, then sceened for their ability to replicate in *Ketogulonigenium* hosts after conjugative plasmid transfer from *E. coli* S17-1 to *Ketogulonigenium* strains ADM 29101 and ADM X6L01 using bi-parental matings.

[0077] A 5 ml overnight culture of each of the *E. coli* S17-1 transformants was prepared in Luria Broth containing 50 ug/ml of kanamycin with orbital shaking at 250 rpm, 37 deg C. Concurrently, 5 ml shaking overnight cultures of strains ADM X6L01 and ADM 29101 (*Ketogulonigenium robustum* and *Ketogulonigenium sp.*, respectively), were prepared in X6L medium containing

50 ug/ml nalidixic acid at 30 deg C. The next day, 50 ul of each *E. coli* culture was transferred into 3 ml of fresh Luria Broth without antibiotic and grown at 37 deg C, 250 rpm until reaching an optical density of 0.1 to 0.4 at 600 nm wavelength. Likewise, 200 ul of each *Ketogulonigenium* culture was transferred to 3 ml of fresh X6L medium without antibiotic and grown at 30 deg C, 250 rpm until reaching an optical density of 0.6 at 600 nm. At this point a bi-parental mating of each *E. coli* culture with each *Ketogulonigenium* culture was prepared. 400 ul of *E. coli* culture was mixed in a sterilized 1.5 ml microcentrifuge tube with 1 ml of *Ketogulonigenium* culture. The mixed cells were pelleted in an Epindorf microcentrifuge and the supernatent was decanted. Cell pellets were suspended in 100 ul of fresh X6L medium and the entire cell suspension was spotted onto a sterile, 0.45 um X 25 mm GN-6 metrical filter from Gelman Sciences (Product No. 63068), which had been placed onto the surface of a fresh X6L agar medium plate. The petri plate and filter with the cells on it was incubated overnight at 30 deg C. The mated cells were then removed from the filter by suspension in 3 ml of fresh of X6L medium. 200 ul of the cell suspension were then plated on fresh X6L medium containing 50 μg/ml kanamycin, 50 μg/ml nalidixic acid, and 1.3% Difco Bacto Agar. The plates were incubated at 30 deg C for two days then inspected.

[0078] Matings between *E. coli* S17-1 hosts harboring plasmid p291-4 and either ADM 29101 or ADM X6L01 recipients produced more than 300 *Ketogulonigenium* transconjugant colonies on the X6L-kanamycin-naladixic acid medium plates. Matings involving any of the other shuttle plasmid candidates produced no colonies with ADM X6L01 recipients, and 3-10 colonies with ADM 29101 recipients. The later may be attributed to recombination of the entering plasmid with the endogenous pADM291 plasmid in the case of ADM 29101 recipients, rather than to maintainance of the incoming plasmid as an independently replicating plasmid.

[0079] The identity of the p291-4 *E. coli/Ketogulonigenium* shuttle plasmid from the ADM 29101 and ADM X6L01 transconjugant strains was confirmed by

restriction digestion and sequencing of plasmid DNA recovered from a transconjugant. The p291-4 shuttle vector was able to be maintained in the *Ketogulonigenium* recipient hosts. The suitability of strains ADM X6L01 and ADM29101 as conjugation recipients demonstrates that both the p291-4 shuttle vector and the transformation method is efficient with multiple species of the genus *Ketogulonigenium*. Methods analagous to those presented in the examples of this invention could be employed to derive additional shuttle vectors for use in *Ketogulonigenium*, for example, from portions of other endogenous plasmids native to other *Ketogulonigenium* strains, including but not limited to endogenous plasmids of the species *Ketogulonigenium robustum* and *Ketogulonigenium vulgarae*. The DNA sequence of PCR product #4, which corresponds to the *Ketogulonigenium* portion of p291-4, is shown in Figure 4 (SEQ ID NO:4).

EXAMPLE 6

Conjugative transfer of shuttle vectors from *E. coli* hosts into *Ketogulonigenium* recipients using tri-parental mating.

[0080] The *E. coli* S17-1 strain that was used as the donor host in the bi-parental mating of Example 5 provides mobilization functions from a chromosomally integrated plasmid. In a tri-parental mating, plasmid-encoded mobilization functions can be provided by a third bacterial strain harboring a broad host range plasmid such as, for example, RP4 (GenBank Accession No. L27758). This would enable a broader range of bacterial strains to be used as the donor host of the shuttle plasmid that is to be conjugated into the *Ketogulonigenium* recipient. An example of a suitable third strain to be used as the source of the mobilization function in a tri-parental mating would be *E. coli* strain HB101 (ATCC 33694) transformed with RP4. An example of a non-mobilizing donor strain harboring the *Ketogulonigenium* shuttle vector would be *E. coli* HB101 transformed with

an *E. coli/Ketogulonigenium* shuttle vector plasmid. The procedure would be the same as in the previous Example, except for the following modification:

[0081] Fresh liquid cultures of *Ketogulonigenium* and the shutlle vector donor strain would be prepared as in the bi-parental mating, except that *E. coli* strain HB101 or another suitable host transformed with the shuttle vector would be used instead of the S17-1 transformant. The third strain, *E. coli* HB101 harboring RP4 for example, would be cultured in suitable medium (Luria Broth containing 5 ug/ml of tetracycline in this example, or a different antibiotic if dictated by the selectted broad host range plasmid), to an optical density of 0.1 to 0.4 at 600 nm. To prepare the mating mixture, 200–400 ul of the two non-*Ketogulonigenium* cultures would each be centrifuged to a pellet in microcentrifuge tubes. The supernatents would be decanted and the pellets would be combined and resuspended in 1.0 ml of fresh X6L medium without antibiotics, then recentrifuged to a pellet and the supernatent decanted again. This washing step in X6L medium is to prevent residual tetracycline from entering the mating mixture. Finally, 1.0 ml of the fresh culture of naladixic acid resistant *Ketogulonigenium* is added and the three cell populations are resuspended together, centrifuged to a pellet, then resuspended in 100 ul of fresh X6L medium and spotted onto the mating filter as in the bi-parental mating. The rest of the tri-parental mating is the same as in the bi-parental mating example.

EXAMPLE 7

Subcloning of p291-4 to make p291-4DS and definition of a pADM291 replicon.

[0082] Example 4 demonstrated that a 2517 bp DNA sequence of endogenous *Ketogulonigenium* plasmid pADM291, corresponding to the DNA sequence of Figure 4 (SEQ ID NO:4), supports the replication of non-native circular DNA molecules in multiple *Ketogulonigenium* species. Upon examination of this sequence using programs of the "Wisconsin Package" (Genetics Computer Group

[GCG], Madison, WI, version 10.1), several open reading frames (ORFs) with protein encoding potential were discovered to exist. These correspond to Figure 2 (SEQ ID NO:2) bases 499 through 1512 for "ORF1", encoding a 337 aa long protein, and bases 1584 through 1979 for "ORF2", encoding a 131 aa long "protein". The predicted amino acid sequences of these proteins was compared against known proteins in the PIR and SWISS-PROT databases using the GCG FASTA and BLAST programs. The results show both to be probable plasmid replication functions, showing the closest homology to other plasmid rep proteins for ORF1, and plasmid resolvase proteins for ORF2. Potential roles for these proteins include plasmid DNA replication, plasmid partitioning or plasmid mobilization, resolution of plasmid multimers, or other functions. The Figure 4 DNA sequence includes non-coding regions of 498 bp upstream of ORF1, 70 bp between ORF1 and ORF2, and 538 bp downstream of ORF2. These non-coding regions could contain one or more plasmid replication origin sites (*ori*V), iterons, sequences encoding antisense RNA encoding sequences, transcriptional promoters, or other features that may participate in control of plasmid replication, partitioning, or copy number. We therefore sought to determine if the *Ketogulonigenium* functional replicon defined by PCR#4 insert of p291-4 could be made smaller by deleting various regions of p291-4 and reintroducing the smaller plasmids into *Ketogulonigenium* by bi-parental mating as in Example 5.

[0083] Plasmid p291-4NX had a 759 bp deletion removing DNA from the *Ketogulonigenium Nhe*I site to the vector polylinker *Xba*I site, leaving only the amino terminus of ORF1 and the noncoding region upstream of ORF1 intact. p291-4ES had a 453 bp deletion removing DNA from the *Ketogulonigenium Eco*RV site to the vector *Sfo*I site, leaving ORF1 and ORF2 and downstream regions intact but removing DNA upstream of ORF1, perhaps including the ORF1 promoter. The plasmid p291-4DS has a 404 bp deletion removing DNA from the *Dra*I site to the *Sca*I site of the *Ketogulonigenium* region, leaving ORF1 and its upstream region and ORF2 intact, but removing some but not all of the DNA downstream of ORF2. p291-4DS is able to be maintained in *E. coli*, be

transferred to *Ketogulonigenium* hosts, and can replicate in *Ketogulonigenium* hosts.

[0084] This Example defines a second *E. coli/Ketogulonigenium* shuttle plasmid, p291-4DS, having the DNA sequence shown in Figure 3 (SEQ ID NO:3). The DNA sequence of the *Ketogulonigenium* portion of p291-4DS, which comprises a *Ketogulonigenium* plasmid replicon, is shown in Figure 1 (SEQ ID NO:1). It is recognized that additional DNA sequences not present in p291-4DS might participate in some aspects of pADM291 plasmid replication, plasmid multimer resolution or other maintainance functions, or plasmid partioning in *Ketogulonigenium* hosts under these or other conditions. If so, it is realized that these functions could be localized and defined using similar methods in accordance with the present invention.

EXAMPLE 8

Transformation of a *Ketogulonigenium* host using electroporation.

[0085] *Ketoguonigenium robustum* strain ADM X6L (NRRL B-21627) was transformed with plasmid p291-4DS using the electroporation method. Competent *Ketogulonigenium* cells were prepared by seeding a single colony of ADMX6L into 10 ml of X6L medium (1% soytone, 1% yeast extract, 0.5% malt extract, 0.5% NaCl, 0.25% K2HPO4, 2% mannitol, pH 7.8). The culture was shaken at 300 rpm at 30 C until reaching an optical density of 0.8 units at 600 nm wavelength. Five ml of this culture was used to seed 500 ml of fresh X6L medium in a 2 L baffled erlenmeyer flask, which was shook at 300 rpm at 30 C until again reaching an optical density of 0.8 units. The culture was chilled in an ice-water bath 10 minutes, then transferd to a pre-chilled centrifuge bottle and centrifuged at 5,000 rpm in a Sorvall 5C-RB Refrigerated Centrifuge for 15 minutes at 4 degrees C. Cells were maintained at 2-4 degrees C for all steps to follow. The supernatent was decanted and the cell pellet suspended in 5 ml

ice-cold Milli-Q water, then additional cold Milli-Q water was was added to a volume of 500 ml. The cells were centrifuged as before, then rewashed in 500 ml of Milli-Q water as before and recentrifuged. The twice-washed cell pellet was suspended in 40 ml of ice-cold 10% glycerol then centrifuged again as before. The supernatent was decanted, then the cells were suspended in a volume of chilled 10% glycerol approximately equal to the volume of the cell pellet. The competent *Ketogulonigenium* cells were aliquoted to microcentrifuge tubes (40 ul per tube) and stored at -80 degrees C.

[0086] A BioRad "Gene Pulser II" electroporator device was set to 1.5 kV, 25 mF. The pulse controller was set to 200 ohms. One ul of p291-4DS DNA prepared as in Example 1 was added to 40 ul of thawed chilled competent *Ketogulonigenium* cells on ice. The cell-DNA mixture was transferred to a pre-chilled electroporation cuvette, which was then transferred to the electroporation device and the pulse was applied. One ml of X6L medium was added to the cuvette, then the mixture was removed, transferred to a 10-ml test tube, and incubated for 2 hours with orbital shaking at 300 rpm and 30 degrees C. The incubated cells (approximately 1.4 ml) were placed in a microcentrifuge tube and spun at 13,000 rpm for 2 minutes, after which 0.9 ml of clear supernatent was carefully removed. The cell pellet was suspended in the remaining supernatent, then the cells were spread onto the surface of an X6L medium agar plate (1.2% Difco Bacto Agar) containing 20 ug/ml of kanamycin, and the plate was incubated for 2 days at 30 degrees C. Colonies that formed on this plate were confirmed to be *Ketogulonigenium* transformed with plasmid vector p291-4DS.

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[0087] All publications mentioned herein above are hereby incorporated in their entirety by reference. All publications cited in the annotations to the Genbank

accession numbers or in ATCC strain descriptions cited herein are hereby incorporated in their entirety by reference.

[0088] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.